Structure of the Single Stable Hemoglobin Adduct Formed by 4-Aminobiphenyl In Vivo

Sir: Aromatic amines are a class of environmental contaminants capable of producing a variety of toxic effects including carcinogenesis and methemoglobinemia (*1–6*). These two end points are often effected by a common metabolic intermediate, the *N*-hydroxylamine. Carcinogenesis is thought to be initiated by the reaction of the electrophilic *N*-hydroxyamine or a conjugate thereof with DNA (*4–6*). In the red blood cell, the *N*-hydroxylamine reacts with oxyhemoglobin through a process in which the oxygen bound to oxyhemoglobin oxidizes both reactants to produce methemoglobin and the arylnitroso intermediate (*1–3*). The arylnitroso compound itself is a highly electrophilic species which reacts preferentially with thiols such as glutathione or the cysteine residues of proteins (*7–12*). Presumably, the thiols add to the nitrogen–oxygen double bond, sulfur attacking at nitrogen, to form a short-lived intermediate which either is reductively cleaved by other thiols to the hydroxylamine or stabilized by oxygen migration from the nitrogen to the sulfur atom to form a sulfinamide (*7–10*).

It has been suggested that the commonality of the mechanism underlying cancer initiation by aromatic amines and the formation of cysteine reaction products might be exploited for the purpose of dosimetry, since protein adducts can be sampled noninvasively and quantified if present in sufficient amounts (*13–15*). We have been exploring this possibility with 4-aminobiphenyl (*16, 17*) which is a human carcinogen (*18, 19*) present in cigarette smoke (*20*). When administered to rats, a particularly high fraction of the dose became covalently bound to hemoglobin, almost all of which was in the form of a single adduct (*16*). All of the evidence indicated that this was a sulfinamide formed at one or more of the available cysteine residues. Facile in vitro hydrolysis of this adduct regenerated the parent amine, allowing detection at the subnanogram level. We also discovered that it was possible to react synthetic *N*-hydroxy-4-aminobiphenyl with human oxyhemoglobin in vitro to obtain a chemically identical adduct at levels as high as one aminobiphenyl residue per tetramer of hemoglobin (*16*). Since in human hemoglobin only the 93β residue reacts readily with thiol reagents such as *p*-mercuriobenzoate (*21, 22*), we were encouraged to crystallize this highly modified protein and determine its structure crystallographically. In this report, we present the structure of human hemoglobin modified by *N*-hydroxy-4-aminobiphenyl as determined by X-ray crystallography, and discuss the effect that the binding of this carcinogen has on the overall structure of the protein.

N-hydroxy-4-aminobiphenyl was prepared by the reduction of 4-nitrobiphenyl as described by Thissen et al. (*23*). It was dissolved in acetonitrile and then added to a 1 mM solution of purified oxyhemoglobin (*24*), at a ratio of 2 equiv of hydroxylamine per tetramer of Hb. The reaction proceeded aerobically with gentle stirring at room temperature for 1 h after which the modified Hb was purified by gel filtration chromatography (Bio Gel P6DG) (*16*). Titration of available thiols with *p*-mercuriobenzoate revealed that 1–1.2 mol of cysteine per tetramer Hb were modified after reaction with *N*-hydroxy-4-aminobiphenyl (*22, 25*). The modified hemoglobin then was treated with a 20-fold excess of sodium nitrite (per tetramer) for 20 min at 37 °C to convert the protein completely to the met form. Excess nitrite was removed by dialysis against 50 mM sodium phosphate buffer, pH 6.8, and the resulting solution was diluted to 4% protein. The hemoglobin sulfinamide adduct and the residual thiol content were determined to be stable toward sodium nitrite treatment based upon methods described in Green et al. (*16*).

Methemoglobin crystals were grown from this preparation by the method of Perutz using a sodium, potassium phosphate buffer with varying concentrations of salts (*26*). The best crystals were obtained from a combination of 1% Hb and a 2.4 M buffer.

Crystals were mounted in sealed glass capillary tubes with a drop of mother liquor. The crystals had a tetragonal lattice with space group $P4_12_12$, $a = b = 54.3$ Å and $c = 197$ Å. The asymmetric unit contains one-half of the hemoglobin tetramer.

Three-dimensional X-ray diffraction data were collected on a Nicolet P-3 diffractometer equipped with a modified LT1 low-temperature device (*27*). Measurements were taken at −15 °C in order to keep radiation damage to less than 25%. A unique data set of reflections to 4-Å resolution was obtained from one crystal measuring 1.5 mm on a side. Each reflection was integrated over the entire ω profile (1.5 Å). A total of 3000 reflections with intensities greater than 1.0 σ were observed. The data were corrected for absorption (*28*) and reduced by standard methods (*29*). Radiation decay was evaluated from a set of five reflections, which were measured every 300 measurements. A linear decay model was fitted to these reflections and used to correct the data.

A difference electron density map was calculated by using phases obtained from the coordinates of human carbonmonoxyhemoglobin (*30*), which also crystallizes in space group $P4_12_12$, $a = b = 54.3$ Å, $c = 193$ Å, and is isomorphous with this derivative. The mean fractional isomorphous difference between the data measured for the derivative and that calculated for native hemoglobin was 12% on F's. The difference electron density map was featureles except for a strong peak (>3σ) near the position of Cys 93β.

A large flat area of electron density and a small additional density were observed connected to the sulfur of Cys 93β. The structure of the ligand and an oxygen atom were fitted into these densities to give the final map. Some movement of the residues around the ligand can be discerned from the appearance of new electron densities in these regions; however, accurate refitting cannot be done at this resolution. Elongation of the C axis in the derivatized hemoglobin is an indication of a change in the packing of the F and H helices. Data to higher resolution could not be obtained because the crystals were very small and reflections, at higher resolution, were not strong enough to be measured accurately.

In Figure 1 the biphenyl group is seen wedged between the F and H helices close to the section where these two helices cross. The predominant interactions between adduct and protein occur along the backbone of residues 141–142 (leucine–alanine, H19–H20) and the side chain of 144 (lysine, HC 1) along one side of the phenyl rings, and residues 86–93 (alanine-cysteine, F2–F9) along the other side. The end of the second phenyl ring shown in Figure 2 is exposed at the surface and makes interactions with groups which are essentially surface groups.

The present work and other previous observations (*8*) indicate that the covalent bond formed between 4-nitrosobiphenyl and cysteine 93β occurs as a final step in the overall binding process. Xenon has been shown to bind to hemoglobin elsewhere than in the distal pocket (*31*), which clearly indicates that noncovalent interactions such as van der Waals forces are adequate to stabilize at least some types of hemoglobin–ligand complexes. The binding site for xenon is in the β subunit near the GH and AB

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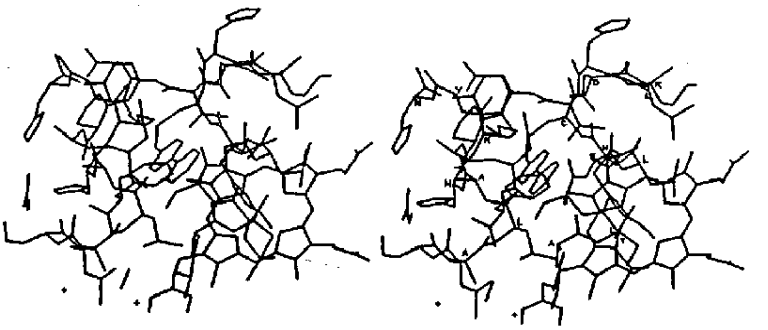

Figure 1. Stereodrawing of the area around the bound biphenyl group. A part of the F helix [residues 86–97: ATLSELHCBKL] runs from bottom to top across the face of the heme. A part of the H helix and C terminus [residues 140–146: ALAHKYH] run from bottom to top toward the upper left corner. The α carbon atoms are labeled with the single letter amino acid code.

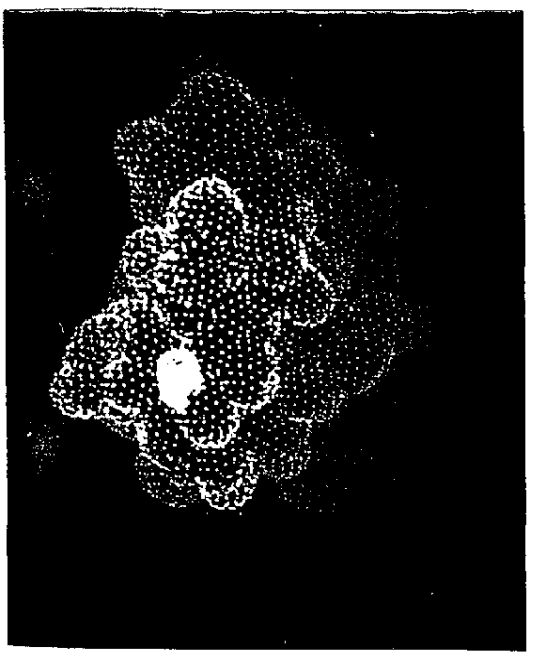

Figure 2. Area accessible (*33*, *34*) to a 1.4-Å probe (water) on the surface of the β subunit derivatized with an aminobiphenyl group. Only the edge of the *p*-phenyl group (white section) is available at the surface.

corners. This is a different site than that in which 4-aminobiphenyl is bound, but it is also in a lipophilic region away from the heme ring.

Further evidence comes from the binding of organic mercurial reagents to hemoglobin thiols. There are six sulfhydryl groups in the human hemoglobin tetramer: cysteines 104α, 112β, and 93β. Of these, the sulfur of 93β reacts readily with mercurial reagents. The other two can be forced into reaction by altering pH and using mercurials with a smaller organic ligand (*32*). Since the sulfhydryl of 93β is completely buried in the native structure, it is very probable that the region in which it occurs is mobile enough to allow access to these organic mercurials. This is in agreement with the present observation that hemoglobin is distorted in the region in which 4-aminobiphenyl is bound. It appears that foreign compounds with considerable diversity of shape can induce structural changes in the protein which allow them to burrow into mobile regions. If one of these regions contains a reactive residue, it may then trap the foreign molecule through covalent bond formation. The absence of stringent shape requirements for the ligands suggests that aromatic amines other than 4-aminobiphenyl may also be substrates for hemoglobin binding.

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Registry No. *p*-$PhC_6H_4NH_2$, 92-67-1; *p*-PhC_6H_4NHOH, 6810-26-0.

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